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p53 is frequently mutated in Burkitt's lymphoma cell lines

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A panel of 12 Burkitt's lymphoma cell lines and four other B cell lines were tested for the presence of mutations in p53. Protein analysis using a mutant-specific antibody and sequencing of both cDNA and genomic DNA revealed changes relative to the standard p53 protein sequence in 12 of the 16 lines studied, including 10 of the BL lines. Mutation of p53 in the BL lines was usually accompanied by loss of the other allele of p53. Testing of the mutated p53 cDNAs for gain of transforming activity or loss of growth suppression activity showed that several of the BL mutants were functionally altered from wild-type p53.
Key words: Burkitt's lymphoma/Epstein-Barr virus/p53/transformation/tumour suppressor

Introduction

Burkitt's lymphoma (BL) is a high grade B cell lymphoma (reviewed in Lenoir and Bornkamm, 1987) which is found world-wide at a low incidence and is also present at a much higher incidence in parts of Africa and New Guinea where malaria is endemic. The tumour is found most frequently in children in Africa. BL cells are characterized by reciprocal chromosome translocations between the c-*myc* locus on chromosome 8 and one of the three immunoglobulin loci (Dalla-Favera *et al.*, 1982). Of these, translocations to the immunoglobulin heavy chain locus on chromosome 14 are the most frequent but translocations to the kappa and lambda light chain gene regions (chromosomes 2 and 22) are also observed. It is presumed that genetic elements within the immunoglobulin loci alter the expression of c-*myc*, thus contributing to the tumour cell growth, although the mechanism and precise effects on c-*myc* expression are incompletely understood. Apart from a slightly different clinical presentation, cases of BL from the high incidence areas are distinguished by usually (~95% of cases; zur Hausen *et al.*, 1970; Geser *et al.*, 1983) carrying Epstein-Barr virus (EBV) in every tumour cell. Most people in the world are infected with EBV, which persists in a small fraction of their B lymphocytes and oropharyngeal epithelium. BL cells from patients in low incidence areas are usually EBV negative although the patients are normally infected with EBV.

The multistep model of carcinogenesis is being confirmed by direct analysis of many tumour types, perhaps the most impressive analysis being that of colon carcinoma (Kinzler *et al.*, 1991). Similarly it is presumed that several oncogenic steps are required for the development of BL; it seems clear that c-*myc* translocation is not the only event since mice transgenic for a rearranged Eμ-*myc* gene were viable and only produced B cell neoplasms at an enhanced frequency, and not polyclonal lymphomas from every B cell (Adams *et al.*, 1985). The established risk factors for BL in Africa of malaria (Burkitt, 1969) and elevated EBV antibody titres (de Thé *et al.*, 1978) may reflect some of these steps.

Changes in the p53 tumour suppressor gene are involved in many different kinds of cancer (Nigro *et al.*, 1989; Prosser *et al.*, 1990; Chiba *et al.*, 1990; Crook *et al.*, 1991b). In those tumour cells, loss of the growth inhibitory (wild-type) p53 function is frequently achieved by point mutation of the p53 protein. Many different point mutations seem to result in approximately equivalent changes in p53 protein function. As a result the mutant p53 loses its tumour suppressor activity and sometimes becomes a dominant oncogene in standard transformation assays (Finlay *et al.*, 1989; Halavey *et al.*, 1990). It is thought that these different point mutations cause a change in conformation of the protein and many of the cancer-associated mutant forms can be detected with a particular monoclonal antibody (PAb240) (Gannon *et al.*, 1990), which fails to recognize wild-type p53. Wild-type p53 appears to function by arresting or slowing the cell cycle at the G_1/S boundary (Michalovitz *et al.*, 1990; Diller *et al.*, 1990) and may be able to inhibit the function or expression of c-*myc* (Finlay *et al.*, 1989; Eliyahu *et al.*, 1989).

In this paper we examine a panel of EBV positive and negative BL cell lines for the presence of p53 mutations. The pattern of p53 mutation and closely linked frequent deletion of the other allele of p53 strongly indicates that mutation of p53 plays a role in the growth of these cell lines.

Results

Mutant p53 proteins in BL cell lines

Normal p53 protein turns over rapidly in cells so it is very difficult to detect by Western blotting. In contrast, the various mutated p53 proteins found in tumours are stabilized in the cell and can accumulate to relatively high levels (Lane and Benchimol, 1990). The BL cell line Ramos was found to contain a much higher level of p53 protein (Figure 1) than two lymphoblastoid cell lines (LCLs, i.e. B lymphocytes immortalized with EBV) IB4 and B95-8. The abundantly expressed protein was considered to be p53 since it bound to two different p53 antibodies, comigrated with p53 which is known to be overexpressed in C33 cells (Benchimol *et al.*, 1982; Crook *et al.*, 1991b) and was not detected when the p53 antibody was omitted from the first stage of the immunoprecipitation/Western blotting procedure. p53 could not be detected in IB4 or B95-8 cells by this procedure, even though an equivalent amount of cell protein had been used in the assays (Figure 1).

The accumulation of p53 has been found usually to reflect

either mutation of the protein or stabilization by binding of p53 to another protein. These alternatives can often be resolved by testing the ability of the p53 to bind to the PAb240 monoclonal antibody, which specifically recognizes a conformation of p53 common to the mutant types. A panel of BL and other B cell lines (Table I) was therefore pulse-labelled with [^{35}S]methionine and cell extracts were immunoprecipitated with the PAb1801, PAb240 or Rb antibodies. PAb1801 (Banks *et al.*, 1986) precipitates both mutant and wild-type p53, PAb240 only precipitates mutant p53 (Gannon *et al.*, 1990) and the Rb antibody served as a control. The p53 protein can be seen in all the cell lines illustrated except Akata using the PAb1801 antibody (Figure 2). The faint bands in Akata around the p53 level are also seen with the anti-Rb antibody and thus represent a background in the immunoprecipitation and not a low level of p53. Only a subset of the lines made p53 protein that bound to the PAb240 antibody; Ramos, Loukces, P3HR1 and Raji were reactive with PAb240. In a separate experiment (not shown) the BL37 cell line also made p53 reactive with PAb240. Some lines gave two bands of p53 and these may correspond to heterozygosity at the common amino acid 72 polymorphism which has been described elsewhere (Matlashewski *et al.*, 1987). Raji give two bands of p53 with PAb1801 but only one of these forms was precipitated with PAb240. These results therefore gave a clear indication that mutant p53 protein is detectable in some but not all BL cell lines.

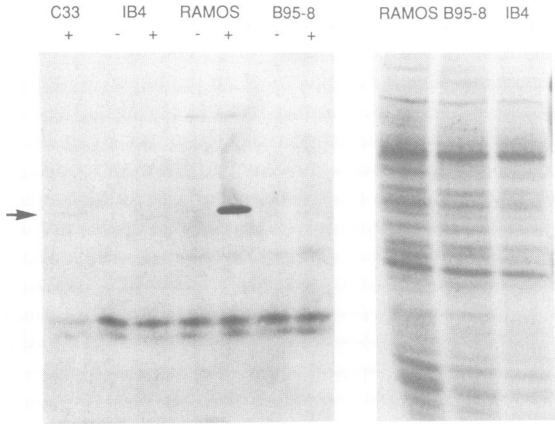

Fig. 1. Accumulation of p53 in Ramos cells. **Left panel**, p53 was immunoprecipitated from cell extracts with PAb1801 (tracks +) electrophoresed on a polyacrylamide gel, Western blotted and the filter probed with a rabbit p53 antiserum. The arrowed band is p53. Mock immunoprecipitations (tracks −) omitting the PAb1801 antibody were processed in parallel as a control. **Right panel**, a portion of the Ramos, B95−8 and IB4 cell extracts used for immunoprecipitation was electrophoresed and stained with Coomassie brilliant blue.

Six of the BL cell lines carry EBV and six do not (Table I). Both EBV positive and EBV negative BLs gave rise to PAb240 reactive p53 so there does not at this level seem to be any simple correlation with EBV status. If an EBV protein were bound at high affinity to the p53 in these cells, it might be expected to appear as an extra labelled band in the PAb1801 or PAb240 immunoprecipitations from the EBV positive cell lines. No such extra proteins were detected (Figure 2) so our results provide no support for binding of an EBV protein to p53, contrary to the situation seen in adenovirus, SV40 and papillomavirus infected cells. The samples were also immunoprecipitated with the PAb 421

Table I. Origins of cell lines, summary of p53 mutations and allelic deletion

Cell line	Ethnic origin	Translocation	EBV	aa72 PM	CB4 mutation	Other mutation	PAb240	Alleles
Burkitt's lymphoma								
IARC/BL2	Caucasian	t(8,22)	−	Arg/Pro	−	−		2
IARC/BL40	Caucasian	t(8,14)	−	Arg/Arg	−	−		2
IARC/BL30	Caucasian	t(8,14)	−	Arg	Met246→Thr ATG→ACG	−		1
IARC/BL41	Caucasian	t(8,14)	−	Arg	Arg248→Gln CGG→CAG	−		1
Loukces	American	t(8,14)	−	Pro	Cys238→Tyr TGT→TAT	−	+	1
Ramos	American	t(8,14)	−	Arg	Ile254→Asp ATC→GAC	−	+	1
Akata	Japanese	t(8,14)	+	Arg	−	190 frameshift GCCCCT→GCCCT		1
IARC/BL37	Caucasian	t(8,22)	+	Arg	Met237→Ile ATG→ATA	−	+	1
Namalwa	African	t(8,14)	+	Arg	Arg248→Gln CGG→CAG	−		1
Raji	African	t(8,14)	+	Arg/Pro	−	Arg213→Gln CGA→CAA	+	2
Jijoye	African	t(8,14)	+	Pro	−	Lys132→Gln AAG→CAG		1
P3HR1	from Jijoye	t(8,14)	+	Pro	−	Tyr163→His TAC→CAC	+	
High grade B lymphoma								
BJAB	African	8q+	−	Arg/Arg	−	His193→Arg CAT→CGT		2
Lymphoblastoid cell lines								
IB4	American		+	Arg	−	−		1
B95−8CR	Caucasian		+	Arg/Arg	−	−		2
EBV converted BL								
BL41-C16	From IARC/BL41		+	Arg	Arg248→Gln CGG→CAG			1

antibody to p53 (Harlow *et al.*, 1981), which recognizes a different epitope on p53 to that of PAb1801 (Arai *et al.*, 1986), but the pattern of precipitation was identical to that seen with PAb1801 (data not shown).

Sequencing of p53 mRNA reveals mutations

To characterize further the likely mutations in p53 detected by protein analysis, cytoplasmic poly(A)$^+$ RNA was prepared from the panel of cell lines. Northern blotting analysis of the p53 mRNA and an approximate quantification relative to actin mRNA showed that all cell lines expressed p53 but at widely differing levels (Figure 3). In general the BL cell lines expressed several-fold more p53 mRNA than the LCLs analysed but the Akata BL line contained a very low level of p53 mRNA.

cDNA was prepared from the poly(A)$^+$ RNA and a 1.3 kb section of the p53 mRNA spanning the coding sequence was amplified by polymerase chain reaction (PCR). All mRNAs gave a major 1.3 kb PCR product of the same size (Figure 4). *Taq* polymerase was used further to copy single-stranded DNA from the PCR products and this was sequenced. The complete coding sequence was established for all the BL p53 mRNAs, the BJAB p53 mRNA and the IB4 and B95-8CR LCL p53 mRNAs. BL41-C16 was only sequenced across conserved box 4. The wild-type protein sequence of p53 and the positions of the DNA primers used for sequencing are shown in Figure 5. The resulting sequences were compared with the standard wild-type DNA sequence determined previously (Lamb and Crawford, 1986). Nucleotide changes and their consequential amino acid changes are summarized in Table I and the sequence changes are illustrated in Figure 6.

The common Arg to Pro polymorphism at amino acid 72 was detected in the mRNA in the Louckes, Jijoye, P3HR1, BL2, Raji and B95-8CR cell lines. The Akata cell line had

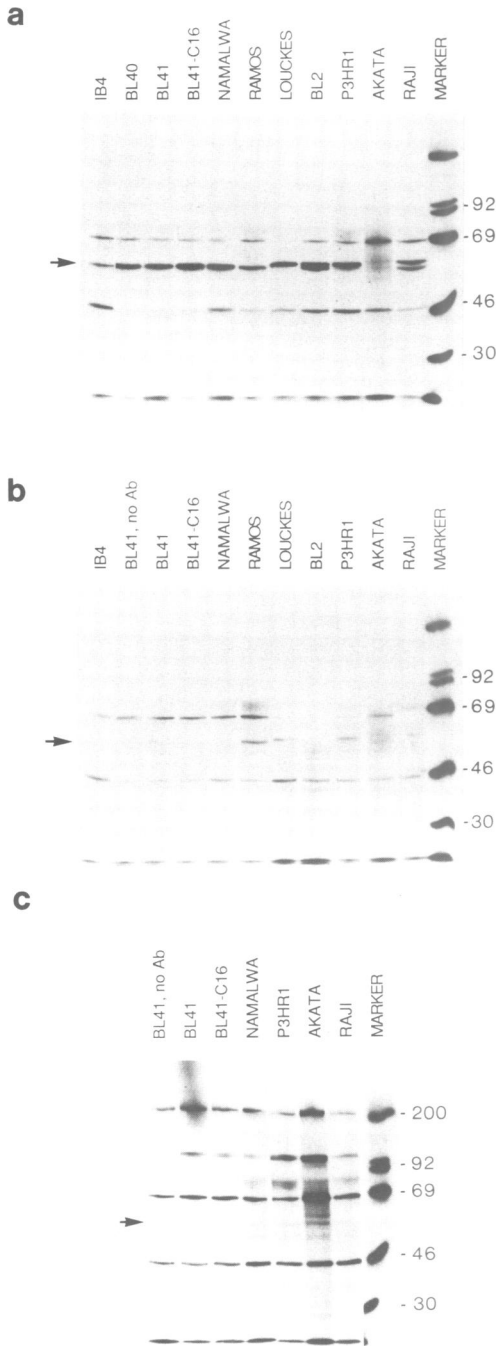

Fig. 2. Pulse labelling p53. Cells were labelled with [^{35}S]methionine and immunoprecipitated with (**a**) PAb1801, (**b**) PAb240 or (**c**) with a rabbit anti-Rb antiserum as a control. The precipitated material was electrophoresed and autoradiographed. The sizes of protein markers are shown in kDa; the arrow is at the position of p53.

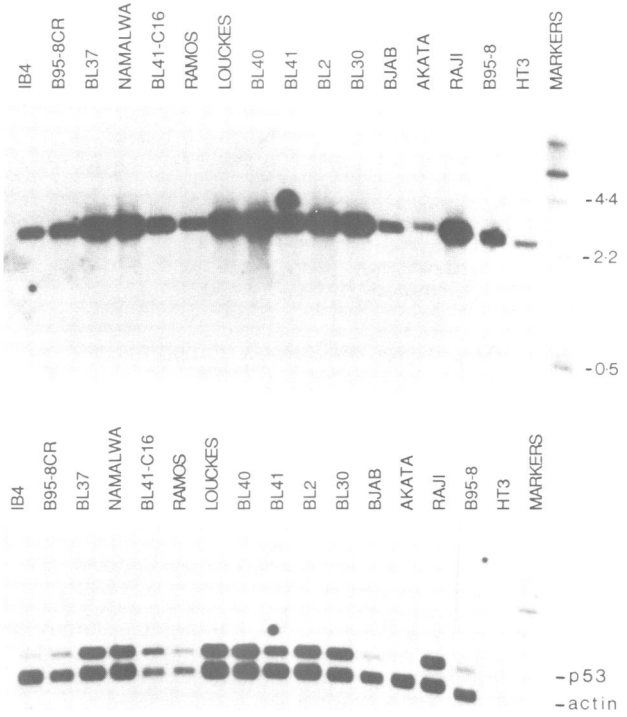

Fig. 3. Northern blot. Poly(A)$^+$ RNA was glyoxalated, electrophoresed and blotted onto nitrocellulose. The filter was hybridized to a p53 probe (**upper panel**) and then rehybridized with an actin probe (**lower panel**) The sizes of the radioactive λ *Hin*dIII DNA size markers are given in kb. HT3 is a cell line whose p53 mRNA has been characterized previously (Crook *et al.*, 1991b) The sizes of RNA coelectrophoresed with these markers can be deduced from the conversion graph in Biggin *et al.* (1987). After densitometry the relative amounts of p53 mRNA in the cell lines (in arbitrary units normalized relative to actin) were: IB4, 5; B95-8CR, 15; BL37, 95; Namalwa, 85; BL41-Cl6, 105; Ramos, 30; Louckes, 70; BL40, 100; BL41, 45; BL2, 75; BL30, 100; BJAB, 10; Akata, 0.2; Raji, 105; and B95-8, 15.

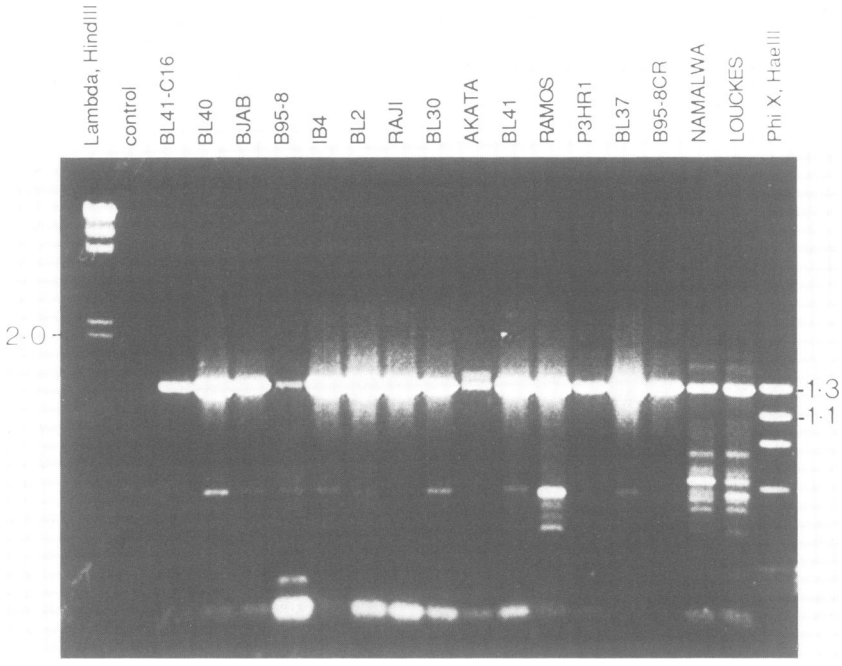

Fig. 4. cDNA PCR products. A portion of the PCR using 216E and 217E on the p53 cDNA was electrophoresed on an agarose gel, stained with ethidium bromide and visualized under UV light. The sizes in kb of some of the size markers are shown.

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          10         20         30         40         50         60         70         80         90        100
                  <box 1>
    MEEPQSDPSV EPPLSQETFS DLWKLLPENN VLSPLPSQAM DDLMLSPDDI EQWFTEDPGP DEAPRMPEAA PRVAPAPAAP TPAAPAPAPS WPLSSSVPSQ
216E>                                                                 <343K                5790>

         110        120        130        140        150        160        170        180        190        200
                  <----------box 2---------->                                    <--box 3--->
  KTYQGSYGFR LGFLHSGTAK SVTCTYSPAL NKMFCQLAKT CPVQLWVDST PPPGTRVRAM AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN
5789>                (218E>)          <344K                     458K>

         210        220        230        240        250        260        270        280        290        300
                                      <--------box 4---------->          <------box 5------>
  LRVEYLDDRN TFRHSVVVPY EPPEVGSDCT TIHYNYMCNS SCMGGMNRRP ILTIITLEDS SGNLLGRNSF EVRVCACPGR DRRTEEENLR KKGEPHHELP
      <5791        6932>     785K>                                                                  <370K

         310        320        330        340        350        360        370        380        390
  PGSTKRALPN NTSSSPQPKK KPLDGEYFTL QIRGRERFEM FRELNEALEL KDAQAGKEPG GSRAHSSHLK SKKGQSTSRH KKLMFKTEGP DSD
                                 (<219E) <369K                                                    <6933<217E
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Fig. 5. Amino acid sequence of p53 (from Tuck and Crawford, 1989) and positions of DNA primers used for PCR and sequencing.

a single base deletion at codon 190 resulting in a frameshift and premature termination after a further 57 amino acids. The resulting protein would probably be unstable in the cell, accounting (with the very low level of p53 mRNA) for the absence of p53 protein in Akata cells (Figure 2). The BL cell lines BL41, Louckes, Ramos, BL30, BL37 and Namalwa all had mutations resulting in amino acid changes within conserved box 4. As expected, the BL41-C16 line (which is BL41 infected with an EBV derived from P3HR1 cells) showed the same p53 mutation as the parent BL41 cells. P3HR1, a cell line obtained by cloning Jijoye showed a mutation relative to Jijoye at codon 163. Raji was mutated at codon 213 and the mutated allele had Arg at codon 72. The method we employed to sequence the PCR copy of p53 mRNA is advantageous because it avoids any cloning of the cDNA so occasional errors made by *Taq* polymerase are averaged out. However, the resulting sequence represents an average of the mRNA sequences present and at this stage it was surprising that the sequence changes observed (Figure 6) were so clear (one might have expected some wild-type RNA also present from the other p53 allele even if one allele were mutated).

Expression of mutant p53 is usually accompanied by loss of the other allele

Evidence for only a single allele of p53 being present in most of the cell lines with mutated genes came from sequencing the genomic DNA covering the points of mutation in conserved box 4 in the p53 genes. DNA from the panel of BL cell lines was PCR amplified in a two stage reaction using the nested primer pairs 218E and 219E followed by 370K and 458K. The resulting 1.35 kb piece covering most of exons 5–8 was copied to single-stranded DNA with 370K and sequenced using primer 785K which is adjacent to

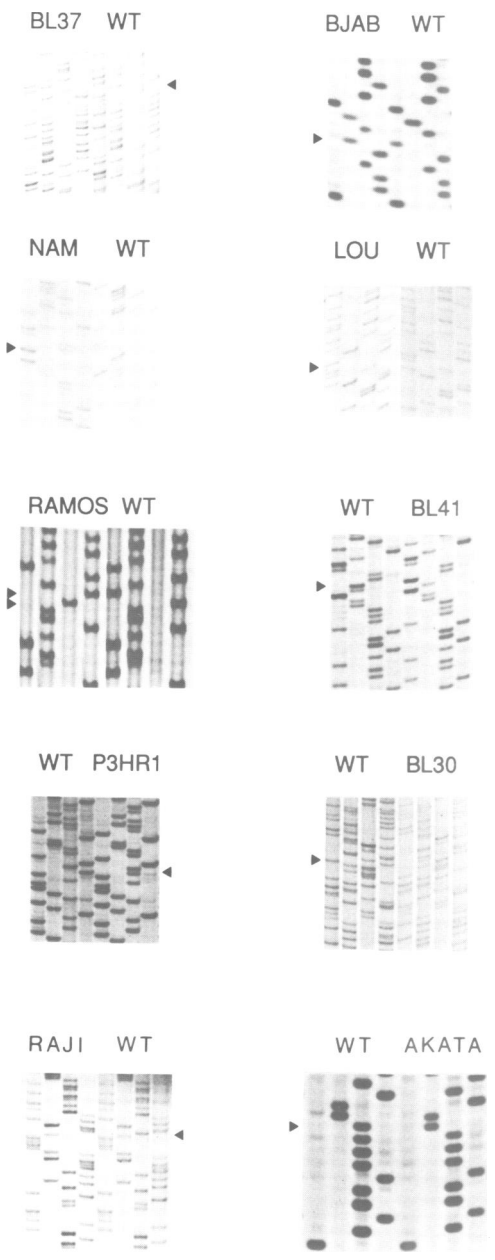

Fig. 6. DNA sequence at points of mutation in cDNA sequences. Lane order is TCGA. The Raji sequence shown is from a clone of the PCR product, the others are direct PCR sequencing. The complete sequences of the cDNAs are available from the EMBL database (Accession numbers X60010 to X60020).

conserved box 4. All the mutations in conserved box 4 (Table I) were confirmed in the genomic DNA and at the point of mutation only a single base was represented (Figure 7). This homozygosity is most easily explained by deletion of the other p53 allele. The cell lines in which no mutation was detected in the cDNA sequencing also show standard sequence on the genomic PCR DNA in the conserved box 4 region, with no indication of heterozygosity. Sequencing the genomic PCR product of Raji across codon 213 showed that Raji is heterozygous at this point, the other allele being wild-type (data not shown).

An alternative method to test for the possibility of allelic loss of the region of chromosome 17 that contains the p53 gene is to probe Southern blots of DNA from the panel of cell lines digested with *Pst*I with the clones p144D6 and pYNZ22.1 (Nigro *et al.*, 1989). These probes hybridize to the 17p13 region where the p53 gene is located and detect restriction fragment length polymorphisms which are very widespread in humans, often giving rise to different sized restriction fragments from the two chromosomes. Results of this type of experiment are shown in Figure 8. Two bands were detected for BL2, BL30, BL40, Raji and BJAB but only one band was observed with both probes for the other DNAs tested. There is a high chance of detecting a difference between the restriction fragment lengths with these two probes if two alleles are present, so it is very likely that most of the single band lines have one allele deleted. Because of the clear single nucleotide at the point of mutation in BL30, it is likely that this also has the other allele of p53 deleted. The two bands detected with probe 144D6 on the Southern blot for BL30 would then imply that one breakpoint of the deletion in BL30 lies between the p53 gene and the 144D6 locus. The data on allelic deletion is summarized in Table I.

Functional analysis of mutant p53 alleles

Wild-type p53 acts as a tumour suppressor gene in appropriate transfection assays; various different mutants of p53 have been observed to lose the suppressor function, to gain a dominant transforming activity or to be unaffected. Most of these studies have used mutant rodent p53 genes, although some mutant human p53 alleles from colon carcinomas have been shown to have gained transforming function (Hinds *et al.*, 1990). We undertook some preliminary assays to test some of the mutant human p53 genes identified in this study for their ability to transform dominantly or to suppress transformation. The p53 cDNAs were all subcloned into a vector which provides a strong promoter (the Moloney murine leukaemia virus long terminal repeat, MoMLV LTR). To test for transforming function, baby rat kidney (BRK) cells were cotransfected with Ha-*ras* in combination with the mutant p53 genes and transformed colonies were scored (Table II). A standard mutant mouse p53 co-operated with *ras* to give colonies and the P3HR1 p53 also gave a few colonies but no other p53 gene gave any colonies. To test for p53 suppressor activity, BRK cells were cotransfected with Ha-*ras* and adenovirus E1a (which co-operate to transform the cells) in combination with the various mutant p53 genes. Parallel experiments were done substituting HPV16 E7 for the adenovirus E1a (the combination of *ras* and E7 also causes transformation). In these suppression assays (Table II) the Ramos and BL37 mutants retained the ability to suppress transformation but the Loukes and BL41 p53s had reduced suppressor activity. The P3HR1 p53 again behaved as weakly transforming in this assay, increasing the number of colonies. As expected, the wild-type p53 gene from BL2 showed normal suppression of transformation. The Raji mutant p53 suppressed transformation by E7 but had lost the ability to suppress E1a; this type of behaviour might be expected of a very weak suppressor since E1a is a more powerful transforming agent than E7 in these assays.

Burkitt's lymphoma biopsies

BL is a relatively rare tumour in the UK and only six frozen tumour biopsies could be located readily. These tumours had all been diagnosed clinically as 'Burkitt's like' but none had been tested for chromosome translocations. DNA was prepared from the tumour biopsies, PCR amplified for the

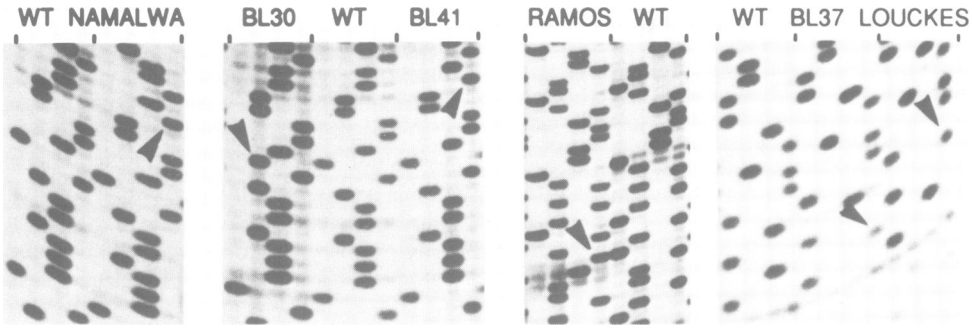

Fig. 7. Sequence analysis of genomic DNA from cell lines at the points of mutation in conserved box 4. Lane order is TCGA.

genomic sequencing as for the cell lines and then sequenced in the conserved box 4 region. No mutations relative to the standard sequence were detected in conserved box 4 (data not shown).

Discussion

Our results revealed, both through protein analysis and sequencing, that most of the BL cell lines studied had mutations in the p53 gene. All of the mutations identified were single nucleotide substitutions except those in Akata (point deletion and frameshift) and Ramos (two adjacent nucleotides substituted). All of the nucleotide changes detected resulted in amino acid changes. The mutations were found spread across the BL cell lines, irrespective of the type of translocation, the ethnic origin of the tumour and the EBV status of the BL cells. No EBV protein was detected associated with p53. All the lines reactive with PAb240 contained mutant p53 but some mutant p53s (including some shown to be functionally altered) did not show PAb240 reactivity. An elevated level of p53 in Raji cells has been reported previously (Benchimol *et al.*, 1982) consistent with the mutation we have characterized.

All of the mutations of p53 we have described are novel except for the Arg248 to Gln present in BL41 and Namalwa. This has been found previously in the MOLT4 cell line derived from an acute lymphoblastic leukaemia (Rodrigues *et al.*, 1990). Other mutations of the same residues have been reported for Met246 (Takahashi *et al.*, 1980), Cys238 (Crook *et al.*, 1991b) and Arg248 (Nigro *et al.*, 1989; Chiba *et al.*, 1990; Hollstein *et al.*, 1990; Malkin *et al.*, 1990; Rodrigues *et al.*, 1990; Stratton *et al.*, 1990). The Arg248 to Gln mutation does not result in a conformation reactive with PAb240 (Figure 2), consistent with the results of Bartek *et al.* (1990) in Namalwa but still appears to cause a reduction in transformation suppression (Table II).

All of the BL lines with mutant p53 except Raji also had the other allele of p53 deleted. This is a frequent phenomenon in other tumours and cell lines that display p53 mutations. In the Akata cell line, no p53 was detected, because one allele was deleted and the other had a frameshift mutation leading to premature termination of the protein.

Since the altered p53 genes in P3HR1, BL41, Namalwa, Raji and Louckes functioned differently from wild-type p53 in our transfection assays, it seems reasonable to conclude that these are significant mutations of the gene that contribute to the growth of the BL cell lines. Similarly the absence of p53 in Akata is likely to be relevant. We have not directly proven that the Louckes and BL41/Namalwa mutant p53

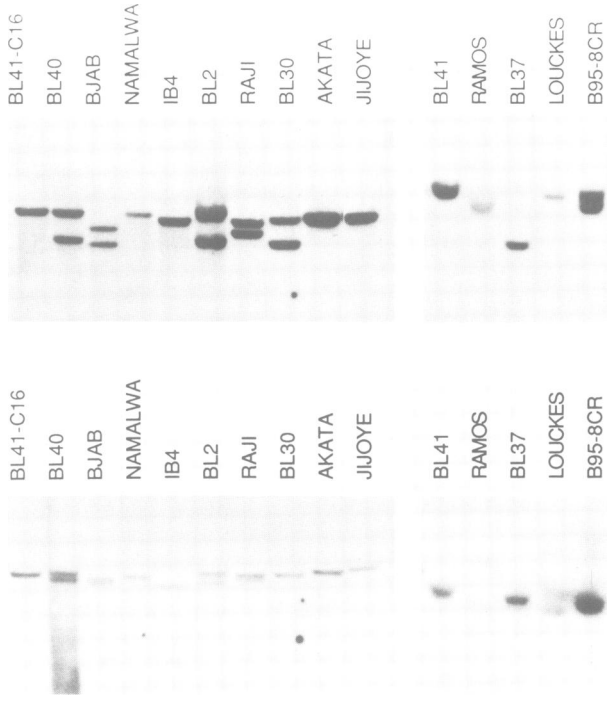

Fig. 8. Southern blots of p53 region of chromosome 17. DNA was digested with *Pst*I and probes were (**upper panel**) p144D6 (Kondoleon *et al.*, 1987) or (**lower panel**) pYNZ22.1 (Nakamura *et al.*, 1988). A separate experiment showed clearly that Louckes gives only one band with pYNZ22.1 (there is a slight smear from the adjacent lane on the filter shown here).

were expressed in the BRK transfections, so their null phenotype could result from lack of expression, but this explanation seems unlikely since all the cDNAs were cloned in an exactly homologous way into the expression vector and had been characterized at the sequence level. At present we have no direct evidence that the mutations in BL37 and Ramos cause functional changes in the protein (because they acted like wild-type p53 in the transfection assays) but the fact that the mutations are within the conserved box 4 region makes it likely that they are significant. The BL cell lines were established from a very wide range of ethnic backgrounds compared with the p53 genes that have been studied previously and the possibility of previously unrecognized sequence polymorphism cannot be discounted; it nevertheless seems unlikely in such a sensitive region of the protein as conserved box 4. The phenotypes seen with the conserved box 4 mutations were fairly weak and this is consistent with current ideas on the box 4 mutations

Table II. Transfection assay of mutant p53 genes in BRK cells

Transfected plasmid (+*ras*)	Exp.1	Exp.2	Exp.3	Mean
pMo	0	0	0	0
p53LTR	16	18	19	17.7
BL2		0	0	0
BL37		0	0	0
BL41	0	0		0
Louckes		0	0	0
Ramos	0	0		0
P3HR1	4	0		2
Raji		0	0	0
E7	27	33	26	28.7
E7+p53LTR	98	107	103	102.7
E7+BL2	0	1	0	0.3
E7+BL37	6	0	0	2
E7+BL41	0	32	22	18
E7+Louckes	26	25	7	19.3
E7+Ramos	3	0	0	1
E7+P3HR1	61	21	30	37.3
E7+Raji	0	2	0	0.7
E1a	30	53	28	37
E1a+pCMVWT	14	7		10.5
E1a+BL2	0	7	3	3.3
E1a+BL37	3	2	11	5.3
E1a+BL41	23	13	19	18.3
E1a+Louckes		14	56	35
E1a+Ramos		2	2	2
E1a+P3HR1	98	17	74	63
E1a+Raji	15	5	28	16

All transfections included the Ha-*ras* gene together with the indicated p53 or control vector. pMo is a control LTR vector, p53LTR is a mouse mutant p53 and pCMVWT is a wild-type human p53 clone (Crook *et al.*, 1991a).

characteristic of the Li Fraumeni syndrome of inherited predisposition to a variety of cancers (Srivastava *et al.*, 1990; Malkin *et al.*, 1990), which are evidently important for tumour development. There is no simple correlation between reactivity with the PAb240 antibody and transformation suppression. Louckes and P3HR1 had much reduced transformation suppression in our assay and were reactive with PAb240; however, Ramos, BL37 and Raji were PAb240 reactive but retained transformation suppression activity.

The principal point of uncertainty in our experiments is whether the p53 mutations and allele loss are a consequence of *in vitro* establishment and culture of the cell lines or were present in the original BL tumours. All the cell lines studied have been in culture for prolonged periods and there is also considerable cell death and selection during the establishment of BL cell lines from biopsies. The six BL tumours we analysed were all wild-type in conserved box 4, implying that the cell line mutations are due to cell culture. However, there are several uncertainties concerning the BL biopsies; their description as 'Burkitt's like' was clinical and histological, no cytological analysis of chromosome translocations could be performed and the proportion of the sample that was actually tumour was difficult to assess. More BL biopsies need to be studied to clarify this issue but at the moment our results tend towards the tumour not carrying the p53 mutations in conserved box 4.

The mutation in P3HR1 p53 is apparently the result of cell culture and cloning since P3HR1 was described as a subclone of Jijoye (Hinuma and Grace, 1967) and Jijoye does not have the mutation at amino acid 163. Jijoye also has a mutation at amino acid 132 which is absent from P3HR1; it would appear that this mutation has also been selected since the cloning of P3HR1. The EBV in P3HR1 has suffered a deletion relative to Jijoye EBV covering the EBNA-2 gene and parts of the EBNA-LP and BHLF1 genes. It is likely that EBNA-2 and EBNA-LP are involved in growth stimulation and immortalization by EBV (Hammerschmidt and Sugden, 1989) so it is interesting to speculate that the p53 mutation might in some way compensate for the loss of those EBV genes. It is noteworthy that the P3HR1 p53 mutation had the strongest phenotype of all the mutations we found, even showing weak transforming activity, which is quite unusual for human p53 mutations. The Daudi BL cell line also happens to contain an EBV with a deletion encompassing the same genes as those missing in P3HR1 but preliminary characterization of its p53 indicates that Daudi has only one allele and that is frameshifted at amino acid 249; the sequence reads AGCCC (data not shown).

The pattern of p53 mutations and allelic deletion combined with the results of functional assays of the mutant p53 genes make it very likely that changes in p53 are important determinants of the growth of some of these lymphoid cell lines. The BL cells all contain translocations of c-*myc* and in Raji alterations of the c-*myc* coding sequence. Analysing the detailed combinations of genetic changes may reveal the mechanism of deregulation of growth in these cells and might yet reveal a contribution of EBV to the growth of the BL cell lines.

Materials and methods

Cell culture

All cells except C33 were maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated fetal calf serum. Prior to labelling experiments, cells were diluted daily to maintain exponential growth and were kept in the range $2-10\times10^5$ cells/ml. The cell lines IARC/BL2, IARC/BL30, IARC/BL37, IARC/BL40, IARC/BL41 (Lenoir *et al.*, 1985), Namalwa (Klein and Dombos, 1973); Louckes (van Santen *et al.*, 1981), Ramos (Klein *et al.*, 1975), Akata (Takada, 1984), Raji and Jijoye (Pulvertaft, 1965) are all derived from BL biopsies. P3HR1 is a subclone of Jijoye (Hinuma and Grace, 1967). BJAB is from an African B cell lymphoma (Menezes *et al.*, 1975). IB4 (King *et al.*, 1980), B95-8CR and B95-8 (Miller *et al.*, 1972) are all LCLs made by immortalizing B lymphocytes with the B95-8 strain of EBV. B95-8 cells are marmoset lymphocytes, all other lines are human. BL41-C16 was made by infecting the BL41 BL cell line with EBV derived from a subclone of P3HR1 (B95-8CR and BL41-C16 were kindly supplied by C.Rooney). C33 cells (a cervical carcinoma cell line; Auersperg, 1964) were maintained in DMEM supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated fetal calf serum.

Cell labelling

15 ml of cell culture in the range $2-8\times10^5$ cells/ml was centrifuged at room temperature for 4 min at 1300 r.p.m. The cells were resuspended in 10 ml RPMI 1640 (no methionine) containing 5% dialysed heat inactivated fetal calf serum. After 30–45 min at 37°C, the cells were again collected by centrifugation and resuspended in 5 ml of the same medium lacking methionine but supplemented with 45 μCi [^{35}S]methionine (1000 Ci/mmol). After labelling for 2 h at 37°C, with occasional inversion to mix, the cells were collected by centrifugation at 4°C and washed twice in ice-cold 150 mM NaCl, 10 mM Tris–Cl pH 7.5. The cell pellet was then lysed in 1.5 ml IP buffer (150 mM NaCl, 50 mM Tris–Cl pH 8.0, 5 mM EDTA, 1 mM PMSF, 1% NP40); the mixture was transferred to a microfuge tube and stood on ice for 20 min. The debris was then removed by centrifugation in the microfuge at 12 000 r.p.m. for 5 min at 4°C. A 2 μl aliquot was counted in a liquid scintillation counter.

Immunoprecipitation of radioactive samples

1.5 ml of cell extract was precleared by addition of 40–60 μl protein G–Sepharose slurry (1:1). After mixing by inversion for 20–60 min at 4°C, the beads were removed by centrifugation. An appropriate amount

of labelled cell extract containing 5×10^7 c.p.m. of ^{35}S was made up to 500 μl with IP buffer. 30 μl of (1:1) protein G–Sepharose slurry was premixed with 15 μl (~1.5 μg) of monoclonal anti-p53 antibody or 5 μl of rabbit anti-human Rb antibody (kindly given by D.Lane). The p53 antibodies 421, PAb1801 and PAb240 were purchased from Oncogene Science. The 500 μl of diluted cell extract was added to this mixture and mixed by inversion overnight at 4°C. The beads were collected by centrifugation and washed three times with 700 μl of IP buffer. The beads were extracted with 100 μl SDS gel sample buffer and a portion of the extracted protein was run on 12.5% SDS–polyacrylamide gels. The gels were impregnated with Enlightening (Dupont) and autoradiographed.

Immunoprecipitation and Western blotting

100 ml of cells at ~1×10^6 cells/ml were centrifuged at 1300 r.p.m. for 4 min at 4°C and the cells washed twice with cold 150 mM NaCl, 10 mM Tris–Cl pH 7.5. The pellet was lysed in 2 ml IP buffer, precleared and immunoprecipitated with the PAb1801 anti-p53 monoclonal antibody, essentially as for the labelled samples, except that all the cell extract was used and the antibody binding was only for 50 min. Half the final immunoprecipitation sample was run on a 10% SDS–polyacrylamide gel and blotted onto nitrocellulose (Rowe *et al.*, 1986). The filter was blocked with milk and probed with a rabbit anti-p53 serum (kindly supplied by D.Lane) using the Amersham ECL detection system.

RNA extraction and Northern blotting

Cytoplasmic RNA was extracted typically from 200 ml of suspension cell culture at ~$5-10\times10^5$ cells/ml. The cells were collected by centrifugation at 1300 r.p.m. for 5 min at 4°C and washed three times with PBS at 4°C. The cells were extracted with two 10 ml aliquots of 150 mM NaCl, 10 mM Tris–Cl pH 7.5, 1 mM $MgCl_2$, 0.1% Triton X-100. These were combined and supplemented with 0.5 ml 0.25 M EDTA, 2 ml 10% SDS and ~10 mg proteinase K. After 5 min at room temperature, the mixture was extracted twice with phenol/$CHCl_3$, once with $CHCl_3$ and ethanol precipitated. The total cytoplasmic RNA was collected by centrifugation and fractionated on oligo(dT) cellulose (Collaborative Research T3) according to Aviv and Leder (1972). The poly(A)$^+$ fraction was ethanol precipitated and redissolved in water. 1–2 μg of poly(A)$^+$ RNA was glyoxylated (McMaster and Carmichael, 1977) and electrophoresed on a 1.2% agarose gel in 10 mM sodium phosphate pH 7.0. After blotting to nitrocellulose (Thomas, 1980), the filter was hybridized with a radioactive p53 probe [p53 cDNA (Lamb and Crawford, 1989), gel purified] labelled by random priming (Feinberg and Vogelstein, 1983). The random priming reaction used 0.1 μg DNA and 50 μCi [α-^{32}P]dCTP. After making several exposures, the filter was rehybridized with a nick translated actin probe (pRT.d3, Biggin *et al.*, 1987) at a lower specific activity. The resulting autoradiograph (made on preflashed film using intensifying screens) was scanned densitometrically. The integrated peak values corresponding to p53 were normalized relative to their actin mRNA signals and converted to arbitary units of p53 mRNA relative to a standard amount of actin mRNA.

DNA extraction and Southern blotting

The nuclear fraction remaining after two extractions of cells with 150 mM NaCl, 10 mM Tris–Cl pH 7.5, 1 mM $MgCl_2$, 0.1% Triton X-100 was resuspended in 100 mM Tris–Cl, 10 mM EDTA, 10 mM Tris–Cl pH 7.5 and supplemented with SDS to 1% and proteinase K to 1 mg/ml. After overnight digestion at room temperature, followed by two phenol/$CHCl_3$ extractions and one $CHCl_3$ extraction, the DNA was ethanol precipitated. The DNA was redissolved in 10 mM Tris–Cl pH 7.5, 1 mM EDTA. After restriction digestion, 5–10 μg DNA was electrophoresed on 1% agarose gels in TBE, blotted to Hybond (Amersham) or nitrocellulose and probed with DNA labelled by the random priming method (Feinberg and Vogelstein, 1983).

cDNA synthesis and PCR of p53 cDNA

cDNA was prepared using the Invitrogen cDNA cycle kit for RT-PCR. Poly(A)$^+$ RNA (0.5–1 μg) was diluted in 10 μl water containing 20 mM methyl mercury hydroxide. After 5 min at room temperature, 2.5 μl 0.7 M 2-mercaptoethanol was added, followed by 1 μl random primers (1 μg/μl), 1 μl placental RNase inhibitor, 4 μl of 5× RT buffer (0.5 M Tris–Cl pH 8.3, 0.2 M KCl, 50 mM $MgCl_2$), 1 μl 25 mM dATP, dGTP, dCTP, dTTP and 5 U of AMV reverse transcriptase (10 U/μl). The mixture was incubated at 42°C for 60 min, then 95°C for 3 min and cooled on ice. A further 5 U of reverse transcriptase was added and incubation continued at 42°C for 60 min. The cDNA was further heated at 95°C, cooled on ice and used directly for PCR. PCR reactions in a volume of 100 μl contained the whole (20 μl) cDNA preparation and 50 mM KCl, 10 mM Tris–Cl pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dGTP, dCTP and dTTP, 50 pmol each of primers 216E and 217E, 1 μl *Taq* polymerase (Amplitaq, Cetus). PCR was for 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2.5 min followed by a final 72°C for 10 min. A 5 μl aliquot was analysed by agarose gel electrophoresis and the resulting 1.3 kb fragment was used either for cloning or asymmetric PCR and sequencing.

Cloning PCR products

A portion of the p53 cDNA PCR 1.3 kb product was end repaired with Klenow DNA polymerase and then digested with *Eco*RI (sites incorporated into primers 216E and 217E). The resulting 1.3 kb *Eco*RI fragment was gel purified and cloned into the *Eco*RI site of pGEM-4Z (Promega). The cloned *Eco*RI inserts were also excised from the pGEM-4Z constructs and subcloned into the *Eco*RI site of pJ4 Omega (Wilkinson *et al.*, 1988); clones were selected with insert orientation appropriate for expression of the p53 gene from the LTR in pJ4 Omega. These clones were used in the transfection assays.

All the pGEM-4Z plasmids were sequenced in the vicinity of the mutation from wild-type p53 to confirm the presence of the mutation.

PCR of p53 from genomic DNA

DNA from cell lines and tumour biopsies was amplified by PCR in a two stage procedure. The first reaction contained ~1 μg genomic DNA in a 100 μl PCR reaction with 50 mM KCl, 10 mM Tris–Cl pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 50 pmol each of primers 218E and 219E and 0.75 μl *Taq* polymerase (Amplitaq). Forty cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 4 min were used followed by a final 72°C for 10 min. A 4 μl portion of the resulting 2.5 kb PCR product was further amplified in an equivalent reaction but substituting primers 370K and 458K and using 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2.5 min followed by a final 72°C for 10 min. This gave a 1.35 kb PCR product covering most of exons 5–8 of the p53 gene.

Asymmetric PCR and DNA sequencing

Asymmetric PCR reactions were used to generate single-stranded DNA for sequencing. Reactions were in 100 μl and contained 50 mM KCl, 10 mM Tris–Cl pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dATP, dGTP, dCTP and dTTP, 50 pmol primer and 0.75 μl *Taq* polymerase (Amplitaq, Cetus) with normally 4 μl of the unpurified product of a previous PCR reaction as template. The p53 cDNA PCRs were copied asymmetrically with either 216E or 217E and the genomic PCRs were copied asymmetrically with either 458K or 370K. PCR was for 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min followed by a final 72°C for 10 min. The whole of the resulting mixture was separated from the mineral oil, extracted with $CHCl_3$, phenol/$CHCl_3$ and ethanol precipitated. The precipitate was collected by centrifugation, redissolved in 30 μl of 10 mM Tris–Cl pH 7.5, 1 mM EDTA and purified by passage through a centrifuged column of Sephadex G-50c in a 1 ml syringe. An aliquot of the resulting solution of single-stranded DNA (~3 μl) could be used directly in DNA sequencing.

Sequencing (by the dideoxynucleotide chain termination method, Sanger *et al.*, 1977) used primers throughout the p53 gene (listed below) and the Sequenase v2.0 kit. Typically 2–3 μl of the purified single-stranded DNA was annealed with 1 pg of primer and then mixed with the standard sequencing reagents, using [^{35}S]dATP and the Sequenase DNA polymerase. Termination reactions were incubated in microtitre plate wells at 37°C for 7 min. The sequencing reactions were fractionated on 6% polyacrylamide gels in TBE and 8 M urea. The entire coding sequence of the p53 cDNA PCR products was determined, usually only on one strand. Any areas of uncertainty were confirmed by sequencing on the other strand and all proposed mutations were determined on both strands. The cell line and biopsy genomic PCR products were only sequenced across conserved box 4, using primer 785K, except for Raji and Jijoye which were also sequenced in the region of their mutations.

Primers used for PCR and DNA sequencing

Many of these primers are similar to those used by Nigro *et al.* (1989) and Bartek *et al.* (1990). Their sequences were as follows: 216 E, GGAATTCCACGACGGTGACACG; 217 E, GGAATTCAAAATGGCAGGGGAGGG; 218 E, GTAGGAATTCGTCCCAAGCAATGGATGAT (in intron 4); 219 E, CATCGAATTCTGGAAACTTTCCACTTGAT (in intron 9); 343 K, AGCAGCCTCTGGCATTCTGGG; 344 K, GGCAGGTCTTGGCCAGTTGGC ; 369 K, TCAGCTCTCGGAACATCTCG; 370 K, GAGGCTCCCCTTTCTTGCGG; 458 K, GCGTCCGCGCCATGGCCATCT; 6932, GGAATTCTAGTGTGGTGGTGCCCTATGAGCCG; 6933, GGAATTCGTGGGAGGCTGTCAGTGGGGAACAA; 5789, GGAATTCTCTGTCCCTTCCCAGAAAACC; 5791, GGAATTCCGAAAAGTGTTTCTGTCATCC; 5790, GGAATTCCAGCTCCTACACCGGCGGCCCCTGCACCAG; and 785 K, GTTGGCTCTGACTGTACCA

Burkitt's lymphoma biopsies

Small frozen biopsy samples were received with summaries of the routine pathology reports. One larger biopsy was a piece of liver containing several metastatic lymphoma deposits. This was thawed and a piece of the white lymphoma dissected out for analysis. The tumour samples were frozen on dry ice, shattered to a fine powder and DNA extracted as for cell lines (omitting the isolation of nuclei). PCR was as for cell line genomic DNA.

Transformation assay in baby rat kidney (BRK) cells

Primary BRK cells were prepared and transfected using calcium phosphate coprecipitation as described previously (Storey *et al.*, 1988; Crook *et al.*, 1991a). 5 μg of each test plasmid was co-transfected with 5 μg pEJ6.6 encoding activated human Ha-*ras* sequences (Shih and Weinberg, 1982) and 1.5 μg pSV2neo. The cells were selected in 300 μg/ml G418 for 2–3 weeks, then scored by microscopic examination or fixed and stained with Giemsa.

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